Copolymers of *N*-isopropylacrylamide can trigger pH sensitivity to stable liposomes

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Abstract Stable liposomes were rendered pH-sensitive by complexation to a polymer that undergoes marked temperature- and pH-dependent water solubility changes. The *N*-isopropylacrylamide-methacrylic acid copolymer was prepared with or without octadecyl acrylate. At pH below the phase transition of the polymer, egg phosphatidylcholine liposomes quickly released a part of their contents only when associated with the octadecyl aliphatic chain grafted polymer at 37°C. Similarly, sterically stabilized liposomes also quickly released a significant part of the entrapped fluorescent markers at pH 5.5–4.9, values corresponding to those of endosomes/lysosomes. This new pH-sensitive liposome-polymer system may further improve the efficiency of liposomal drug delivery.
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Key words: Liposome; Drug delivery; pH-sensitive release; Lower critical solution temperature polymer; Poly(*N*-isopropylacrylamide); Poly(ethylene glycol) derivative

1. Introduction

Numerous studies have reported on the development of pH-sensitive liposomes as drug delivery systems [1]. Since liposomes are internalized by cells mainly via the endocytic pathway [2], pH sensitization of liposomes is an attractive strategy to facilitate the delivery of membrane-impermeable drugs in the cytoplasm before lysosomal enzymatic degradation occurs. Unsaturated phosphatidylethanolamine (PE) has been widely employed to confer intrinsic pH sensitivity to liposomes because it undergoes bilayer-to-hexagonal (H$_{II}$) phase transition upon acidification of the external aqueous medium [3]. Although such liposomes have been shown to be efficient systems for cytoplasmic delivery in cultured cells [1], their moderate stability and rapid blood clearance have hampered their in vivo use. Colloidal stabilization of liposomes can be improved by inclusion of ganglioside (GM$_1$) or poly(ethylene glycol)-derivatized lipids (PEG-PE) [4]. These so-called sterically stabilized liposomes (SSL, Stealth) have shown long circulation half-lives, reduced uptake by the mononuclear phagocyte system and accumulation in tumors [4,5]. Such coating of PE-based pH-sensitive liposomes increases their stability and circulation time in blood but simultaneously reduces their pH sensitivity [6,7]. To circumvent this drawback, the use of cleavable PEG coating has recently been proposed [8].

Acid-triggered liposome destabilization/fusion can be achieved extrinsically by using non-peptidic titratable synthetic polymers [9,10]. The advantage of this approach is the potentiality to render different lipid-based formulations sensitive to pH, without the limitations associated with PE-based liposomes. Although fusogenic peptides can also trigger membrane disruption at acidic pH and have been successfully used to enhance the transfection efficiency of plasmid DNA [11], they display several disadvantages in the development of pH-sensitive liposomes, including high cost of production, immunogenicity and non-trivial association to the liposome surface. Several recent studies have shown that liposomes coated with copolymers of *N*-isopropylacrylamide (NIPA) bearing alkyl chains acquire thermo-responsive properties [12–14]. The alkyl substituent can interact strongly with the liposome membrane and serves as anchor for the polymers onto the liposomes [15,16]. The homopolymer of NIPA is physically characterized by its lower critical solution temperature (LCST), which is around 32°C in aqueous solutions [17,18]. The polymer is soluble below its LCST and separates from solution above it. This temperature sensitivity was used to destabilize the lipid bilayer of liposomes and to induce the release of their contents in response to an increase in external temperature [12–14]. By randomly introducing a small amount of a pH-sensitive monomer in the structure of poly(NIPA), it is possible to increase its LCST above 37°C and make the polymer pH-responsive [19–21]. This property was, for instance, exploited in the preparation of pH-sensitive hydrogels containing crosslinked copolymers of NIPA for the controlled delivery of low molecular weight compounds [22] and macromolecular drugs [23,24]. In the present study, we demonstrate that copolymers of NIPA, methacrylic acid (MAA) and octadecyl acrylate (ODA) can trigger pH sensitivity to egg phosphatidylcholine (EPC) liposomes and SSL.

2. Materials and methods

2.1. Chemicals
NIPA, MAA and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Eastman Kodak (Rochester, NY). NIPA was purified before use, following the procedure described by Gehrke et al. [25]. MAA was distilled at 55°C and 73 cm Hg vacuum before use. AIBN was dissolved in ethanol, filtered, recrystallized in water and dried under vacuum. ODA was obtained from Aldrich (Milwaukee, WI) and used as received. EPC, cholesterol (Chol) and PEG-PE

($M_{rPEG} = 2000$) were from Avanti Polar Lipids (Alabaster, AL). Fluorescent markers were obtained from Molecular Probes (Eugene, OR).

2.2. Synthesis, molecular weight and phase transition determination of copolymers

NIPA, MAA, ODA (94:5:1 or 95:5:0 molar ratio) and AIBN (0.12% m/v) were dissolved in distilled 1,4-dioxane. The dioxane mass was 11 times the total monomer mass. The solution was degassed by bubbling N_2 for 15 min and then heated under stirring at 65°C for 15 h. Polymers were recovered by precipitation in diethylether, resolubilized in tetrahydrofuran, reprecipitated and extensively washed with diethylether. Polymers were then dried under vacuum for 5 days. The average molecular weights of polymers were determined by gel permeation chromatography as previously described [26]. Monodisperse polystyrene standards were used for calibration. The weight average molecular weights of poly(NIPA-co-MAA) and poly-(NIPA-co-MAA-co-ODA) were 8730 and 9760, respectively. Phase transition pH of polymers was determined by 90° light scattering ($\lambda_{ex} = \lambda_{em} = 450$ nm) after 5 min incubation at 37°C in MES buffered saline (100 mM MES, 2 mM EDTA, 144 mM NaCl) of pH values ranging from 4.7 to 6.3. Assuming that the initial molar ratio of each component is preserved after polymerization, the number of anchors per polymer chain (N) can be calculated using the following equation:

$$N = (M_{wPOLYMER} \cdot f_{ODA})/(M_{wNIPA} \cdot f_{NIPA} +$$
$$+ M_{wMAA} \cdot f_{MAA} + M_{wODA} \cdot f_{ODA}) \qquad (1)$$

where $M_{w\alpha}$ and f_{α} are the molecular weight and the molar fraction of comonomer α respectively, and $M_{wPOLYMER}$, the molecular weight of the copolymer.

2.3. Liposome preparation, characterization and leakage assay

Unilamellar liposomes (20 mM) composed of either EPC or EPC/Chol/PEG-PE (3:2:0.3 molar ratio) were prepared by the reverse-phase evaporation method [27] followed by repeated extrusion through 0.1 μm pore membrane [28]. Encapsulation of fluorescent markers into liposomes was performed by using an isotonic aqueous solution of HPTS-DPX-HEPES (35 mM-50 mM-20 mM) pH 7.2. Untrapped dye was removed by gel exclusion chromatography on Sephadex G-50. Phospholipid concentrations were determined by phosphate assay [29]. Liposomes were mixed with polymers and gently stirred overnight at 4°C to form liposome-polymer complexes. The mean diameters of EPC liposomes and SSL were 160 ± 40 nm and 125 ± 30 nm, respectively, as determined by dynamic light scattering (Coulter N4 Particle Size Analyzer) and remained unchanged by the presence of the polymer at the polymer/lipid ratios tested. 10 μl of the complex (corresponding to 280 μg of lipid) was added to 2 ml of buffer and the release of liposome contents was monitored by fluorescence dequenching assay using liposomes with encapsulated HPTS-DPX. The extent of contents release was calculated from excitation fluorescence intensity of HPTS at $\lambda_{ex} = 413$ nm after a 5 min exposure to different pH at 37°C (pH-independent isosbestic point, $\lambda_{em} = 512$ nm [30]) over that obtained after sample lysis in 0.1% (m/v) $C_{12}E_8$ (100% release). Zeta potentials were derived from electrophoretic mobility measurements in 2 mM Tris-HCl, pH 8, containing 10% sucrose (m/v), using a Zetasizer 4 (Malvern Instruments, Ltd., UK) after adjustment to a negatively charged standard (AZ55, Malvern).

3. Results and discussion

Fig. 1 shows the effect of pH on the solubility of poly(NIPA-co-MAA) and poly(NIPA-co-MAA-co-ODA) in buffer. Both copolymers exhibit a discrete phase transition (cloud point) between pH 5.7 and 5.1 at 37°C. These polymers are soluble above pH 5.7 and start to precipitate as pH decreases, the phase transition being fully reversible. It should be noted that the pH range where the phase transition of these polymers occurs is similar to that of the endosomal/lysosomal compartments of the cell [31]. It has been shown that the incorporation of a small fraction of an ionizable comonomer
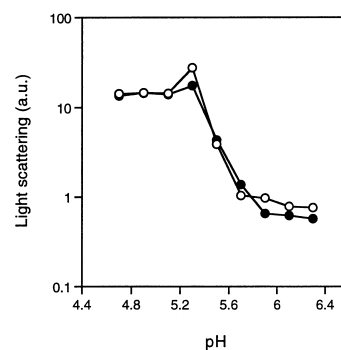

Fig. 1. Solubility of poly(NIPA-co-MAA) (closed circles) and poly(-NIPA-co-MAA-co-ODA) (open circles) at 37°C in MES buffer, as a function of pH. Polymer concentration was 45 μg/ml.

in the structure of poly(NIPA) can lead to a LCST that becomes sensitive to pH [19,21]. At neutral pH, the carboxylic groups of MAA are ionized and the LCST is shifted above 37°C because of the higher overall hydrophilicity of the copolymer as compared to the homopolymer of NIPA. At acidic pH the protonation and removal of charge from MAA brings the LCST back to a value below 37°C, and induces the precipitation of the polymer, which is predominantly driven by the hydrophobic interactions between the isopropyl side groups of NIPA [18,22]. The presence of 1 mol% ODA, which was added to allow the anchoring of the polymer to the liposomes, does not change the phase transition pH of poly(NIPA-co-MAA). The hydrophobic substituent may not be exposed to water but rather forms a micellar structure protected from water by the poly(NIPA) chains, and therefore does not make a hydrophobic contribution to the phase transition pH [15].

The pH-triggered release of fluorescent markers from liposomes and liposome-polymer systems after a 5 min incubation at 37°C is presented in Fig. 2. The decrease in pH from 7.2 to 4.9 produces a 10-fold increase in the total amount of dye released from EPC liposomes associated with poly(NIPA-co-MAA-co-ODA) (Fig. 2A, c,d). Interestingly, preincubation of liposomes with the same polymer lacking the alkyl chain does not induce pH-triggered liposomal leakage (Fig. 2A, b). This indicates that the presence of alkyl chains in the structure of the copolymer is essential for liposome pH-dependent response, possibly due to efficient complexation of the polymer to the liposome membrane via octadecyl chains, as previously shown for similar systems [14,15]. Acidic-mediated liposome leakage was only slightly enhanced (about 15%) by increasing the ODA-containing polymer to lipid mass ratio by a factor of two (Fig. 2A, c,d), suggesting a binding saturation process of the polymer to the liposome surface. We noticed that after addition of the alkyl chain grafted polymer to EPC liposomes, the samples remained transparent, whereas increased turbidity of liposome suspensions without polymer occurred in time over a period of a few weeks at 4°C, presumably due to liposome aggregation (not shown). This phenomenon of stabilization over time was not observed upon addition of the copolymer lacking alkyl chains. Moreover, it has been reported that liposomes coated with copolymers of NIPA show reduced liposome interaction with plasma proteins including opsonins [15]. These findings are consistent with a possible liposome stabilizing effect of such polymers. Stability in plasma and pharmacokinetics of poly(NIPA-co-MAA-co-ODA)-

liposome complexes are currently under investigation in our laboratory.

It was recently shown that SSL containing specific ligands at their surface are efficiently internalized by target tumor cells whereas SSL lacking the targeting device are not [32,33]. Therefore, it appears attractive to confer additional pH-sensitive properties to internalizable SSL formulations for tissue-specific intracytoplasmic drug delivery in vivo. Experiments on contents release with SSL show results similar to those obtained with EPC liposomes (Fig. 2B). The presence of cholesterol, and more importantly, the incorporation of 6 mol% PEG-PE, do not seem to prevent contents release upon acidification and in the presence of poly(NIPA-co-MAA-co-ODA). This is supported by electrophoretic mobility data showing that zeta potential of liposome-poly(NIPA-co-MAA-co-ODA) complexes (polymer/lipid = 0.28 m/m, 0.02 mol/mol) are about −15 mV for EPC and −6 mV for SSL-associated polymer, respectively, whereas EPC and PEGylated liposomes show a neutral particle surface. This difference in zeta potential can be explained either by shielding of the polymer charge by PEG at the surface of the complex [34], or by reduced binding efficiency of polymer to PEG-coated liposomes.

The release of HPTS from SSL after 5 min at low pH is about 15% lower than for EPC liposomes in the presence of poly(NIPA-co-MAA-co-ODA) at both polymer to lipid ratios tested (Fig. 2). The release kinetics of HPTS from EPC and PEGylated liposomes complexed to poly(NIPA-co-MAA-co-ODA) show that 15–20% of the liposome contents are released within 30–40 s irrespective of the liposome lipid composition (Fig. 3). After 5 min of treatment at acidic pH (4.9), EPC complexes have reached their plateau for contents leakage, whereas SSL complexes have not (Fig. 3). It seems that the stabilizing effect of PEG-PE (or the presence of cholesterol

Fig. 3. Release kinetics of fluorescent marker from liposomes at 37°C in the presence of poly(NIPA-co-MAA-co-ODA) with polymer/lipid = 0.28 (m/m). (a) EPC polymer at pH 4.9; (b) SSL polymer at pH 4.9; (c) SSL polymer at pH 5.5.

or both) slows down the release of fluorescent markers from liposomes. Furthermore, contents release from SSL increases with decreasing pH within the transition pH range of the polymer (Fig. 2B, c and Fig. 3, b,c). Importantly, liposome destabilization starts at pH values corresponding to those of endosomes. Incubation time of SSL with poly(NIPA-co-MAA-co-ODA) is an important parameter influencing the efficiency of the polymer to induce pH sensitivity to SSL. Indeed, the addition of liposomes and polymer to the acidic medium without prior incubation did not show significant acid-mediated release of HPTS (5.8%). However, premixing of SSL with this copolymer for 90 s led to a 10% contents release which is about half that obtained after an overnight incubation at 4°C.

pH-triggered release of contents from liposomes by poly(NIPA-co-MAA-co-ODA) may result from a transient destabilization of the liposome membrane due to the conformational change of the polymer upon acidification. Moreover, charge neutralization by protonation of the polymer carboxyl groups renders the polymer more hydrophobic and more susceptible to interact strongly with the lipid bilayer, causing membrane structure defects. A possible partial withdrawal of the anchor groups from the lipid bilayer upon contraction of the polymer [35] may also be involved. Several hypotheses can be considered to explain that only a fraction of the liposome contents could be released (20–25%). Perturbation of the lipid membrane could be temporary, due to fast rearrangements of lipid molecules after the collapse of the polymer has occurred, stopping further liposome leakage. There also may be only some liposome populations containing sufficient amounts of polymers to induce release of fluorescent markers. A tendency of hydrophobically modified copolymers of NIPA to form polymer-rich domains and phase separation of the contracted polymer at the liposome surface have been reported by others [36], suggesting that events such as pore-like formation can also be involved in the mechanism of release.

Membrane-anchored ionic polymers have previously been employed to induce liposome destabilization and/or fusion but showed disadvantageous requirements such as very large polymer concentration [9] and/or very high charge density of the polymers [9,10] to obtain the desired effect. Moreover, it has been shown that the pH sensitivity of PE-based liposome formulations decreases with increasing amount of either cholesterol [37] or PEG-PE [7]. Our studies indicate that poly(NIPA-co-MAA-co-ODA) confers pH sensitivity to liposomes
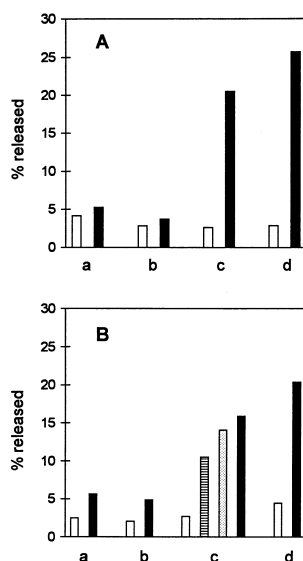

Fig. 2. Release of entrapped fluorescent marker after 5 min incubation at 37°C from EPC liposomes (A) and sterically stabilized liposomes (B). (a) Control liposomes; (b) liposomes in the presence of poly(NIPA-co-MAA), polymer/lipid = 0.28 (m/m); (c) liposomes in the presence of poly(NIPA-co-MAA-co-ODA), polymer/lipid = 0.28 (m/m); (d) liposomes in the presence of poly(NIPA-co-MAA-co-ODA), polymer/lipid = 0.56 (m/m). Open bars, pH 7.2; closed bars, pH 4.9; hatched bar, pH 5.3; gray bar, pH 5.5. Values for duplicates varied than less than 8%.
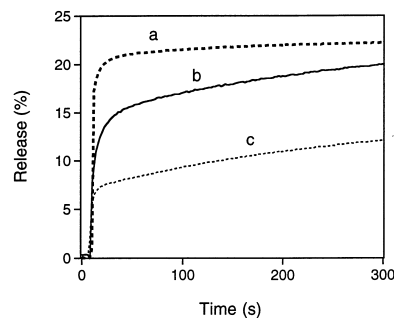
containing up to 40 mol% cholesterol and 6 mol% PEG-PE, with characteristics for pH-triggered release similar to other systems previously described [6,7]. Surface charged liposomes can be potentially rendered pH-responsive by such copolymers, however, electrostatic repulsion between poly(NIPA-*co*-MAA-*co*-ODA) and negatively charged liposomes can alter their association efficiency. For cationic liposomes, the association with anionic copolymers of NIPA may be restricted to a certain range of polymer/lipid ratios where aggregation and/or precipitation of polymer-liposome complexes does not occur. Since the number of anchors per polymeric chain was determined to be less than one ($N = 0.86$, see Eq. 1 in Section 2), one can expect an improved pH-mediated liposome leakage if N is increased. Resolution of this issue, as well as the influence of the molecular weight of the polymer on the release of liposome contents, require further study. This promising pH-sensitive system may lead to a more efficient intracytoplasmic drug delivery in vivo.

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